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13. ABSTRACT ***(Maximum 200 Words)***

The overall goal of this project is to understand the mechanism of regulation of human BRCA2 gene expression in order to explore the possibility of epigenetic malfunction in this mechanism, which may lead to sporadic breast cancer. BRCA2 mRNAs were only detected in dividing cells but not at all in quiescent cells. We have found a transcriptional silencer at the upstream of human BRCA2 gene. This silencer is active only in the quiescent cells but not in the dividing breast cells, thus explaining the absence of BRCA2 mRNA in the quiescent cells. The mechanisms of the activation and inactivation of this silencer in the quiescent and dividing cells, respectively, are presently unknown. We have shown that specific nuclear proteins from quiescent breast cell nuclear extract sequence-specifically binds to this silencer. We also have observed that at least some of the African-American breast cells may have alteration in this regulatory pathway. We hypothesize that the human BRCA2 gene is silenced in the quiescent stage of breast cells but is activated in the dividing cells by the inactivation of the silencer. Possible transient epigenetic malfunction in this silencer inactivation process by environmental factors in the dividing cells may lead to defect in DNA repair and subsequence onset of mutations in any key gene leading to oncogenesis. Our studies may relate this regulatory pathway with respect to the ethnic origin of the breast cells.

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Introduction

The overall goal of this proposed project is to understand the mechanism of regulation of human BRCA2 gene expression in order to explore the possibility of epigenetic malfunction in this mechanism, which may lead to sporadic breast cancer. The majority (>95%) of human breast cancer happen sporadically and caused by mutations in a variety of genes (1-5). On the other hand, the familial breast cancers are caused by the defects in either of the two DNA repair protein genes, BRCA1 and BRCA2 (1). Possibility of epigenetic malfunction in the expression of these genes in developing sporadic breast cancer has been proposed. BRCA2 mRNAs were only detected in dividing cells but not at all in quiescent cells (1-5). Recently, we have found an Alu-repeat containing transcriptional silencer at the upstream of human BRCA2 gene (6). This silencer is active only in the quiescent cells but not in the dividing breast cells, thus explaining the absence of BRCA2 mRNA in the quiescent cells. The mechanisms of the activation and inactivation of this silencer in the quiescent and dividing cells, respectively, are presently unknown. We have shown that specific nuclear proteins from quiescent breast cell nuclear extract sequence-specifically binds to this silencer (6). Understanding the structure-activity relationships in these bindings in reference to covalent modifications of the DNA elements and the protein factors may reveal the mechanisms of the regulation of the silencer function. Thus, we believe that the human BRCA2 gene is silenced in the quiescent stage of breast cells but is activated in the dividing cells by the inactivation of an Alu-containing silencer located at the upstream of the BRCA2 gene promoter. Possible transient epigenetic malfunction in this silencer inactivation process by environmental factors in the dividing cells may lead to defect in DNA repair and subsequence onset of mutations in any key gene leading to oncogenesis. Since there are indications that the development and progression of breast cancer in African Americans may be different from that of Caucasians (7-16), we planned to explore whether the BRCA2 silencer turn-on and turn-off mechanisms are altered in the breast cells isolated from African American females.

Task 2: **To identify the sequence elements in the BRCA2 gene silencer responsible for the activity of the silencer by mutational analysis.** (This was our planned commitment for Y02)

Identification of the regions of the 221 bp silencer that are occupied by the silencer binding proteins: We have tentatively identified four possible regions of importance in the 221 bp silencer region (Fig. 1). There are two Alu sequences (Alu1 and Alu2) and two corresponding non-canonical E-box sequences (E1 and E2). We performed some DNase I foot-printing analysis with the 221 bp silencer DNA as probe. Data (Fig. 2) show perhaps global binding of breast cell nuclear proteins throughout the silencer.

Site-directed mutagenesis of the potential cis-elements in the BRCA2 silencer: The QuikChange site-directed mutagenesis kit (Stratagene) was used. We designed the mutagenic primers according to the instruction given by Stratagene in there manual to alter key nucleotide sequences known to be essential for the functions of Alu and E-box elements. The lists of the mutagenic primers are given in Table 1. The 221 bp silencer was cloned into pCRII vector (Invitrogen) for mutagenesis. After mutagenesis the insert in the mutated plasmid DNA was sequenced to verify mutagenesis and then the mutated silencer was subcloned into the reporter

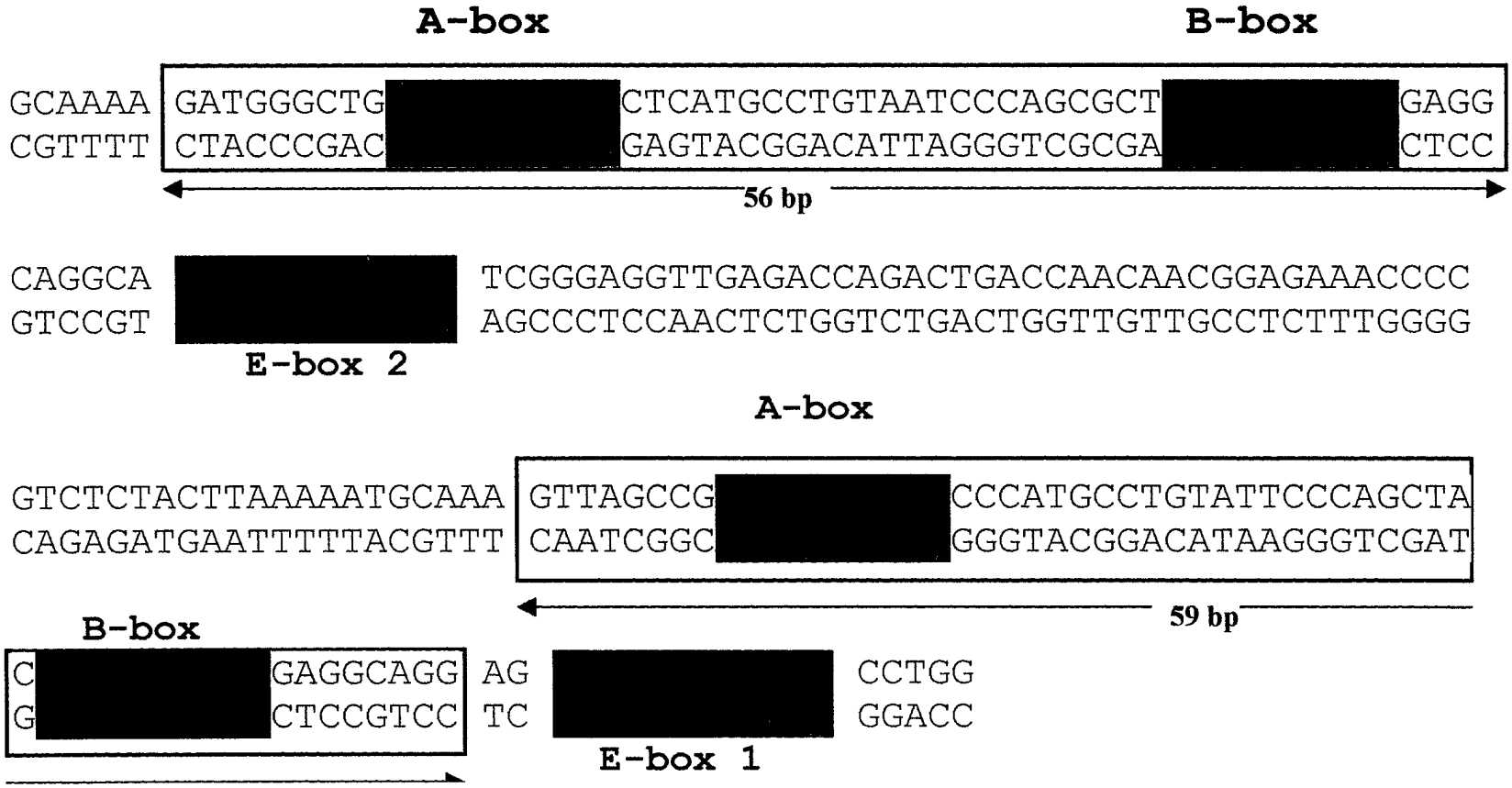


Fig. 1. Nucleotide sequence of the 221 bp silencer showing the putative cis-elements.

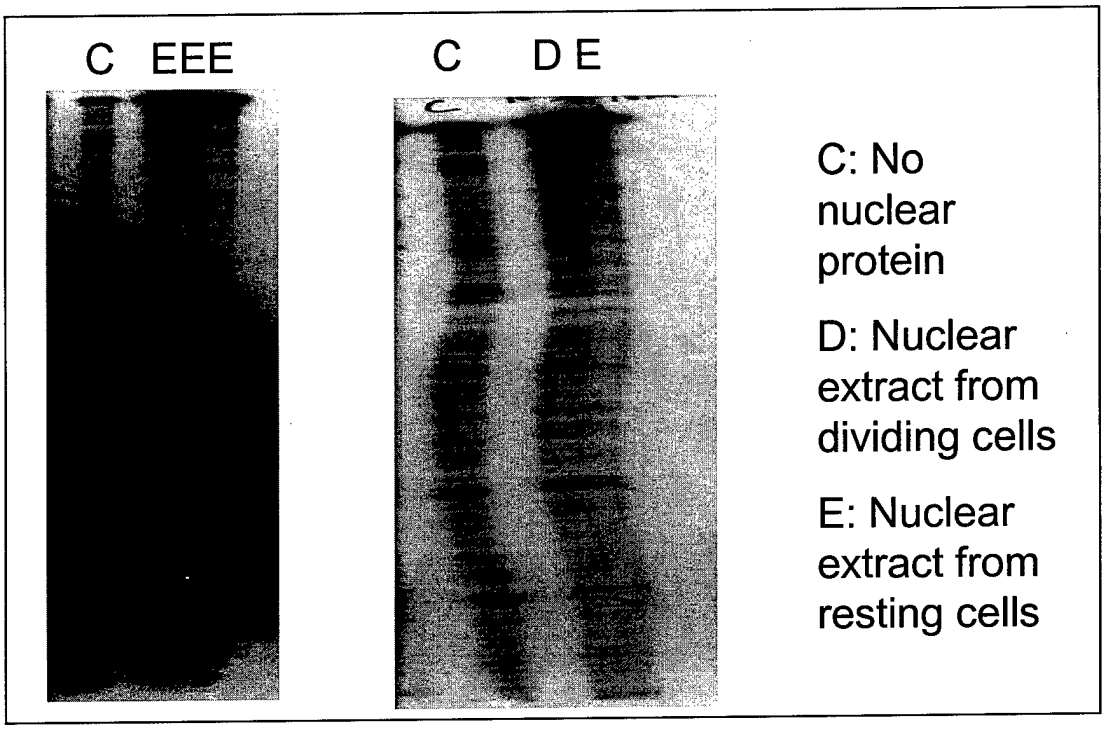
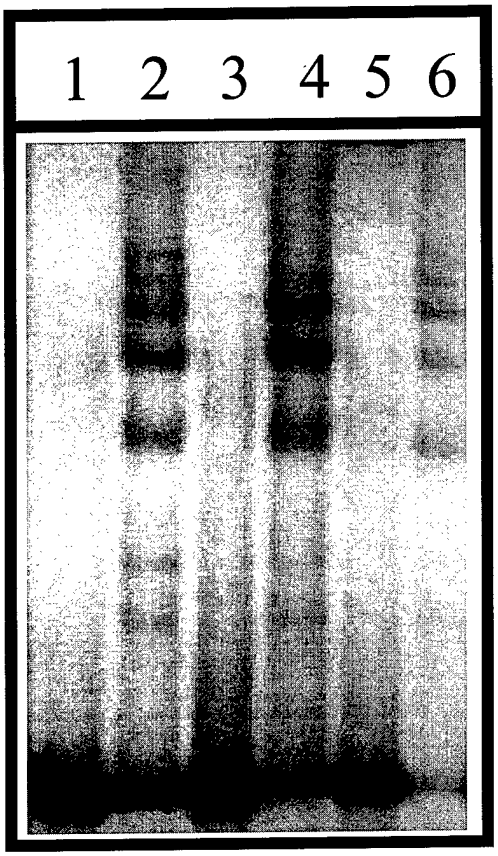


Fig. 2. DNase I foot-printing analysis of the binding of proteins affinity-purified from the nuclear extract from MDA-MB-231 cells with the 221 bp silencer sequence. Proteins were purified by binding with a biotin-labeled 221 bp silencer sequence. Preparation equivalent to 10^6 cells were used per incubation. There was no detectable protein in the prep from dividing cells.

Table 1. Primers used for Site Directed Mutagenesis (SDM): Mutant nucleotides are in bold and underlined.

E-box 1 (E1) primers	
EB1P1M	5'-GGCAGGAGAACCACT**GA**ATCCCTGG-3'
EB1P2M	5'-CCAGGGAT**TC**AGTGGTTCTCCTGCC-3'
E-box 2 (E2) primers	
EB2P1M	5'-CAGGCAGATCACC**GA**AGGTCGGGAGG-3'
EB2P2M	5'-CCTCCCGAC**CT**TCGGTGATCTGCCTG-3'
Alu 1 primers	
Alu1P1M	5'-CCAGCTACTCGG**CGA**GCTGAGGCAGG-3'
Alu1P2M	5'-CCTGCCTCAGC**TCG**CCGAGTAGCTGG-3'
Alu 2 primers	
Alu2P1M	5'-CAGCGCTTTGG**CGA**GCCGAGGCAGG-3'
Alu2P2M	5'-CCTGCCTCGGC**TCG**CCAAAGCGCTG-3'



Lane 1: No Extract
Lane 2-6: With nuclear extract

Lane 2: No competitor
Lane 3: E1 competitor
Lane 4: Mutated E1 comp.
Lane 5: E2 competitor
Lane 6: Mutated E2

Fig. 3. Autoradiogram showing binding of nuclear proteins from MDA-MB-231 cells to 221 bp human BRCA2 silencer. All incubations had poly(dI-dC). There was 50-fold excess of the competitor DNA.

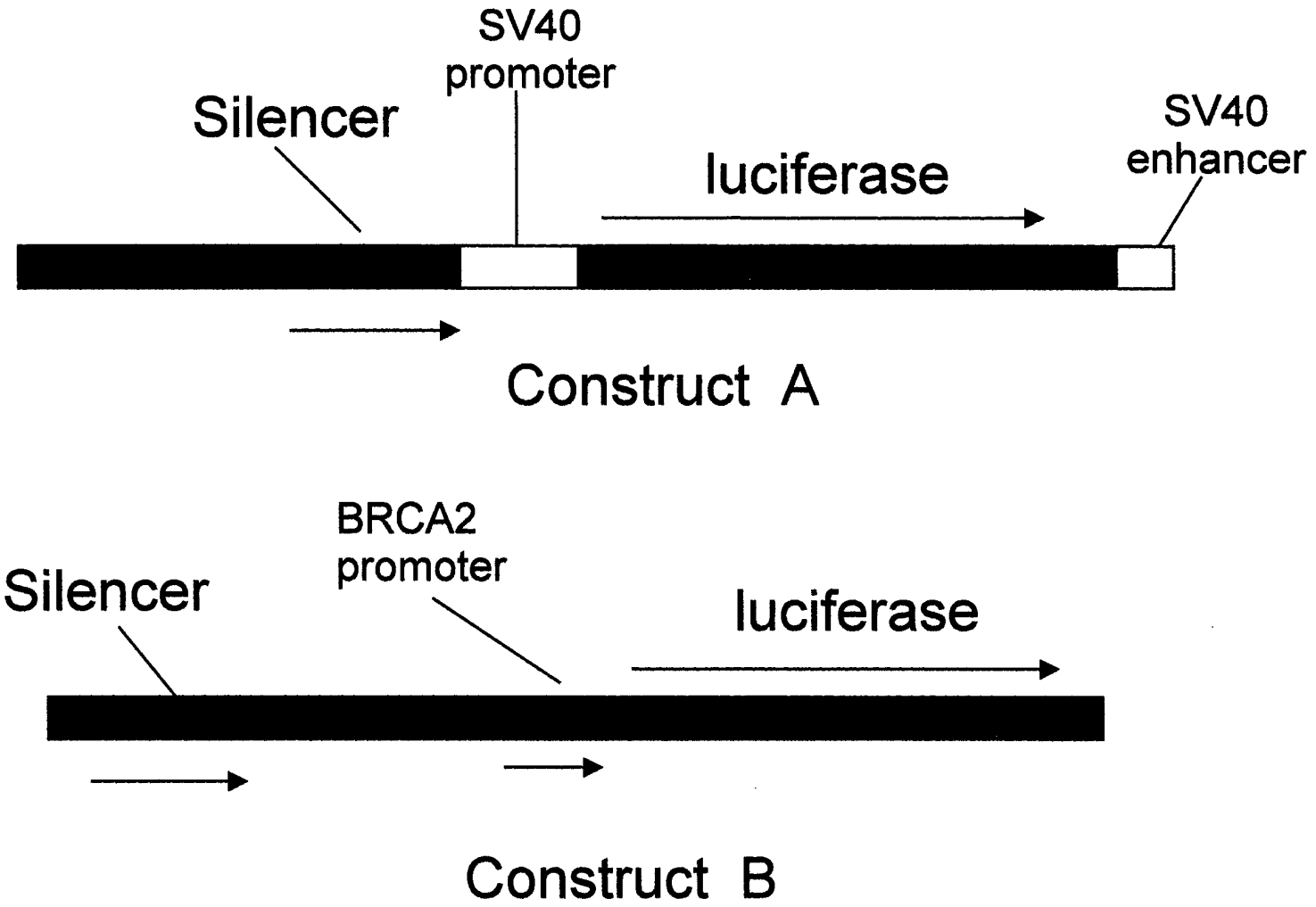


Fig. 4. Reporter plasmid constructs (pGL3 background) to test the structure-activity relationships of the silencer sequence.

Table 2. Effects of mutations in the cis-elements present in the BRCA2 silencer on the silencer activity inside MDA-MB-231 cells. (Data are Mean for four independent experiments ± SE)

Mutated locus	Silencer activity (% with no mutation)
None	100
Alu1	46.3 ± 1.7
Alu2	44.2 ± 2.1
E1	90.1 ± 2.3
E2	96.2 ± 1.2
Alu1+Alu2	42.7 ± 2.5
E1+E2	88.7 ± 1.3
Alu1+E1	58.1 ± 1.1
Alu2+E2	61.3 ± 2.8
Alu1+E2	23.2 ± 0.9
Alu2+E1	30.2 ± 1.2
Alu1+Alu2+E1+E2	0.00

Data are shown with plasmid construct B. Similar results were obtained with plasmid construct A.

vector (pGL3; see Fig. 4). We are successful in obtaining four single mutants, six double mutants and one mutant having all the four cis-elements mutated (see Table 2 for their names).

Evaluation of protein binding to the mutated silencer: We determined the binding of breast cell (MDA-MB-231) nuclear proteins to the silencer and determined the competition of those bindings with wild-type and mutated cis-element sequences (ds-DNA composed of annealed oligos as detailed in Table 1). While Alu1 or Alu2 sequences did not show any detectable competition of protein binding to the silencer (data not shown), E1 and E2 sequences competed for the bindings (Fig. 3). Mutated E-box sequences failed to compete (Fig. 3) for the binding, suggesting thatthese elements are important for the protein binding at least in vitro. We have repeated this experiment with nuclear extracts from MDA-MB-468, BT-549 and MCF-7 cells which gave us similar data (not shown).

Evaluation of the silencer activities of the mutants: We have tested the activities of the control and mutated silencer sequences against SV40 promoter/enhancer or BRCA2 promoter/enhancer systems (Fig. 4). Our results are summarized in Table 2. Mutations of either of the Alu elements abrogated the silencer activity somewhat. On the other hand mutations of either of the E-box sequences alone did not have any effect on the silencer activity. Mutants containing Alu1+E2 or Alu2+E1 mutations were found be largely inactive as silencer. Mutations in all the four cis-elements completely abrogated the silencer activity (Table 2).

Key Research Accomplishments

- We found four major cis-elements in the 221 bp silencer sequence and developed mutant constructs to test structure activity relationships.
- The silencer binding proteins in nuclear extracts from the breast cells seem to bind to the non-canonical E-box sequences in the silencer, at least in vitro.
- Mutations in the Alu elements along with E-box sequences appears to be lethal for the silencer activity

Reportable Outcomes

None yet.

Conclusions

The negative transcriptional regulator present at the upstream of human BRCA2 gene seems to require certain cis-elements to function. Blocking of those elements, either by sequestration with proteins or covalent modifications inside breast cells may thus have potential to regulate BRCA2 gene expression.

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